

Allozyme analysis of Japanese *Semisulcospira* species (Gastropoda: Pleuroceridae) reveals that Lake Biwa endemic species are not monophyletic*

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Abstract: Lake Biwa (Shiga Prefecture, Japan) is an ancient lake with many endemic species. In particular, 15 extant endemic species of the genus *Semisulcospira* (Caenogastropoda: Pleuroceridae) have attracted attention because they may represent a single species flock. Although many taxonomic studies have been conducted on these Lake Biwa species and on non-Biwa species of the genus, their genetic relationships remain unresolved. We use allozyme analysis to investigate the genetic differentiation of 12 species of the genus *Semisulcospira*, including nine Lake Biwa endemics: *S. (Biwamelania) arenicola* Watanabe and Nishino, 1995, *S. (B.) decipiens* (Westerlund, 1883), *S. (B.) fluvialis* Watanabe and Nishino, 1995, *S. (B.) multigranosa* (Böttger, 1886), *S. (B.) nakasekoe* Kuroda, 1929, *S. (B.) ourense* Watanabe and Nishino, 1995, *S. (B.) fuscata* Watanabe and Nishino, 1995, *S. (B.) niponica* (Smith, 1876), and *S. (B.) habei* Davis, 1969, and three non-Biwa species: *S. (S.) libertina* (Gould, 1859), *S. (S.) reiniana* (Brot, 1877), and *S. (S.) kurodai* Kajiyama and Habe, 1961. Based on their genetic features, the Lake Biwa endemics were divided into three groups, the *S. (B.) decipiens* group, *S. (B.) niponica* group, and *S. (B.) habei* group. The observed genotype frequencies of the Lake Biwa endemics within each group were similar to each other. We tested the observed genotype frequencies for each polymorphic locus in each local population of the Lake Biwa endemics for departure from Hardy–Weinberg equilibrium. Most of the eight polymorphic loci of the Lake Biwa populations did not deviate significantly from HWE, except for Aat of *S. (B.) arenicola* at the site B8, Pgm-1 of *S. (B.) fluvialis* at the site B9, and *S. (B.) habei* at the site B1 (level of significance $\alpha = 0.05$). The *S. (B.) niponica* and *S. (B.) habei* groups showed similar genetic features to the non-Biwa *S. (S.) libertina* and *S. (S.) reiniana* groups, whereas the *S. (B.) decipiens* group was distinct from any other group. Our allozyme data suggest that *Semisulcospira* species in Japan are distinguished robustly into two major lineages, the *S. (B.) decipiens* group and a separate non-*decipiens* group, and that all extant Lake Biwa endemics do not form a single species flock, as proposed previously.

Key words: genetic variation, allozymes, Lake Biwa, endemic speciation, *Semisulcospira*

Lake Biwa (Shiga Prefecture, Japan) has endured for about four million years and is recognized as an ancient lake, similar to Lake Tanganyika in Africa and Lake Baikal in Russia. Many endemic species in several taxa—for example, of shrimp, fish, and molluscs—have been reported in Lake Biwa (Mori and Miura 1980), as they have in other ancient lakes. In particular, 15 endemic species of the subgenus *Biwamelania* in the genus *Semisulcospira* (Caenogastropoda: Pleuroceridae) have been investigated because they may represent a single species flock (Nishino and Watanabe 2000). However, the genetic diversity of these Lake Biwa endemics has not been studied adequately. It is important to consider the genetic relationships among the Lake Biwa endemics to understand their speciation pattern during the formation of

the Lake Biwa water system, and to help elucidate the reasons why so many endemic species have been maintained in the lake. In this study, we investigated the genetic variation of Japanese *Semisulcospira* species including nine Lake Biwa endemics, using allozyme analysis.

Davis (1969) studied the taxonomy of Japanese *Semisulcospira* species and divided them into two groups: the *S. libertina* species complex and the *S. niponica* species complex, based on the number of embryos in the female brood pouch, basal cords of adult shells, and chromosomes. The *S. libertina* species complex contains *S. libertina* (Gould, 1859), *S. reiniana* (Brot, 1877), and *S. kurodai* Kajiyama and Habe, 1961, which live in a widely dispersed area outside Lake Biwa. The *S. niponica* species complex consists of six Lake

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Biwa endemic species, *S. niponica* (Smith, 1876), *S. decipiens* (Westerlund, 1883), *S. multigranosa* (Böttger, 1886), *S. reticulata* Kajiyama and Habe, 1961, *S. nakasekoe* Kuroda, 1929, and *S. habei* Davis, 1969. Habe (1978) classified the “*S. niponica* species complex” under the genus *Biwamelania*, and Matsuoka and Nakamura (1981) assigned it to the subgenus *Biwamelania* with *S. niponica* as a holotype. Watanabe (1984) described *S. morii*, which lives only around small islands in Lake Biwa. Watanabe and Nishino (1995) investigated morphological variation in the endemic species and proposed eight new species: *S. fuscata*, *S. dilatata*, *S. rugosa*, *S. ourense*, *S. takeshimensis*, *S. shiraishiensis*, *S. fluvialis*, and *S. arenicola*, classified under the subgenus *Biwamelania*. Moreover, they reported that *S. niponica* contains three morphological types, the *biwae*, *nodose*, and *ribbed* types, based on their shell sculptures. Consequently, it was suggested that many more species than previously thought are endemic to Lake Biwa.

As taxonomical research has improved, variation in shell morphology of *Semisulcospira* species has been clarified. Urabe (1992) studied two sympatric species, *S. libertina* and *S. reiniana*, from the viewpoint of genetic and morphological variation, and determined that they could be divided into two populations having unique homozygous genotypes at the locus *Mpi*: types *Mpi*-A and *Mpi*-B. She also suggested that it is difficult to identify the two species based only on their shell morphology and pointed out the individuals with the *Mpi*-B genotype could be assigned to *S. reiniana*. Recently, Kamiya and Shimamoto (2005) confirmed that the *Semisulcospira* populations with the *Mpi*-A genotype from Tohoku (northern area of Japan) to Kansai District (western area of Japan) correspond to *S. (S.) libertina*, and that the populations having the *Mpi*-B genotype in Kanto (eastern area of Japan) to Kansai District are *S. (S.) reiniana*.

A study of the phylogeny of *Semisulcospira* species was conducted by Oniwa and Kimura (1986). They examined the phylogenetic relationships of six species: *S. (S.) libertina*, *S. (S.) reiniana*, *S. (S.) kurodai*, *S. (B.) niponica*, *S. (B.) multigranosa*, and *S. (B.) nakasekoe*, through allozyme analysis, and noted large genetic differences between the Lake Biwa endemics, *S. (B.) niponica*, *S. (B.) multigranosa*, and *S. (B.) nakasekoe*, and the non-Biwa species, *S. (S.) libertina*, *S. (S.) reiniana*, and *S. (S.) kurodai*. However, the phylogenetic positions of these endemic species remain unsettled.

The fossil record of *Semisulcospira* has been studied by Matsuoka (1987). The fossils of *S. (B.) habei* occur from the Katata Formation (early to middle Pleistocene), and this species is assumed to be the oldest extant species of *Semisulcospira* (Matsuoka 1987). Nishino and Watanabe (2000) presumed that *S. (B.) habei* is a common ancestor of all extant Lake Biwa endemics, and that these endemic

species were derived over a short period of time from *S. (B.) habei*.

We investigated the genetic variation and relationship among nine endemic species of the subgenus *Biwamelania*: *Semisulcospira (B.) niponica*, *S. (B.) decipiens*, *S. (B.) multigranosa*, *S. (B.) nakasekoe*, *S. (B.) fuscata*, *S. (B.) ourense*, *S. (B.) fluvialis*, and *S. (B.) arenicola* in Lake Biwa; and three non-Biwa species of the subgenus *Semisulcospira*, *S. (S.) libertina*, *S. (S.) reiniana*, and *S. (S.) kurodai*, which are widely dispersed outside Lake Biwa. Using allozyme analysis, we found that the endemic species of the subgenus *Biwamelania* can be discriminated into three groups, the *S. (B.) decipiens* group, *S. (B.) habei* group, and *S. (B.) niponica* group. The *S. (B.) habei* and *S. (B.) niponica* groups are closely related with the non-Biwa species *S. (S.) libertina*, *S. (S.) reiniana*, and *S. (S.) kurodai*, whereas the *S. (B.) decipiens* group is significantly different in its genetic features and phylogenetic position.

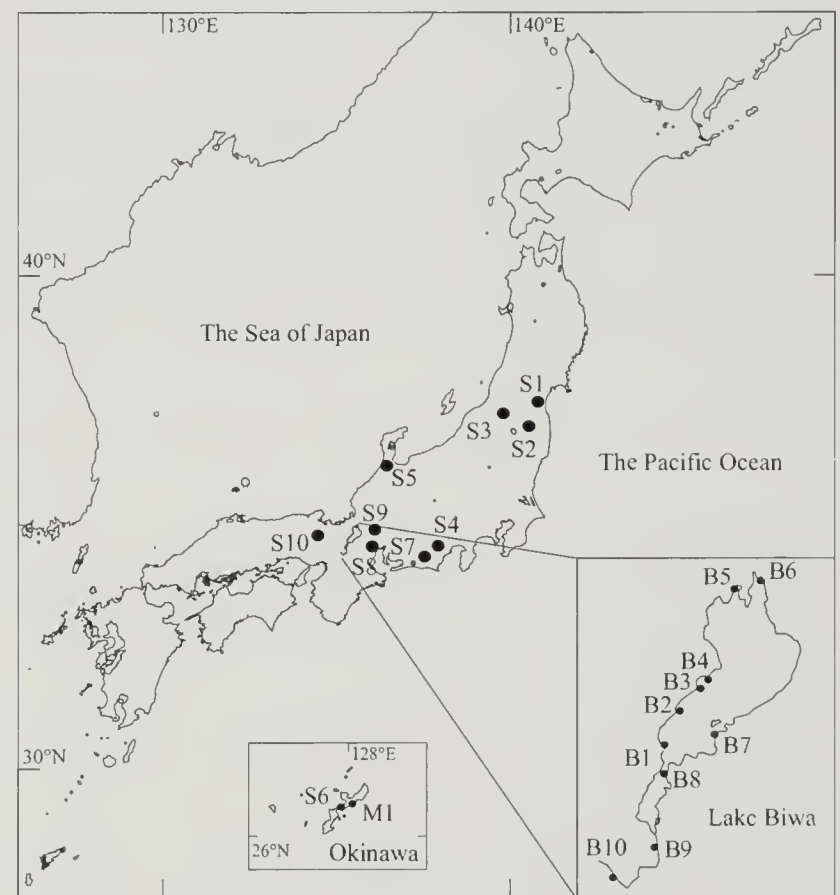


Figure 1. Map of the Japanese Islands indicating sample collection sites. S1, Sendai (Miyagi); S2, Fukushima (Fukushima); S3, Sekikawa (Niigata); S4, Fujieda (Shizuoka); S5, Kanazawa (Ishikawa); S6, On-na (Okinawa); S7, Hamamatsu (Shizuoka); S8, Komaki (Aichi); S9, Takatomi (Gifu); S10, Yoka (Hyogo); B1, Wanihama (Lake Biwa); B2, Omimaiko (Lake Biwa); B3, Shirahigehama (Lake Biwa); B4, Haginohama (Lake Biwa); B5, Oura (Lake Biwa); B6, Hannoura (Lake Biwa); B7, Mizugahama (Lake Biwa); B8, Imahama (Lake Biwa); B9, Nango (Seta River); B10, Uji (Uji River); M1, Nago (Okinawa).

Table 1. Sample collection sites and sample size of local populations, with voucher material number of the IGPS of studied specimens.

Site	Species	Locality	N	Inventory number
B8	<i>Semisulcospira</i> (<i>Biwamelania</i>) <i>arenicola</i> Watanabe and Nishino, 1995	Imahama, Lake Biwa	50	IGPS No. 110201
B2	<i>S. (B.) decipiens</i> (Westerlund, 1883)	Omimaiko, Lake Biwa	27	IGPS No. 110202
B3	<i>S. (B.) decipiens</i> (Westerlund, 1883)	Shirahigehama, Lake Biwa	45	IGPS No. 110203
B4	<i>S. (B.) decipiens</i> (Westerlund, 1883)	Haginohama, Lake Biwa	15	IGPS No. 110204
B9	<i>S. (B.) fluvialis</i> Watanabe and Nishino, 1995	Nango, Seta River	21	IGPS No. 110205
B5	<i>S. (B.) fuscata</i> Watanabe and Nishino, 1995	Oura, Lake Biwa	52	IGPS No. 110206
B1	<i>S. (B.) habei</i> Davis, 1969	Wanihama, Lake Biwa	16	IGPS No. 110207
B2	<i>S. (B.) habei</i> Davis, 1969	Omimaiko, Lake Biwa	18	IGPS No. 110208
B4	<i>S. (B.) multigranosa</i> (Böttger, 1886)	Haginohama, Lake Biwa	35	IGPS No. 110209
B10	<i>S. (B.) nakasekoe</i> Kuroda, 1929	Uji, Uji River	48	IGPS No. 110210
B7	<i>S. (B.) niponica</i> (nodose type) (Smith, 1876)	Mizugahama, Lake Biwa	50	IGPS No. 110211
B6	<i>S. (B.) niponica</i> (ribbed type) (Smith, 1876)	Hannoura, Lake Biwa	50	IGPS No. 110212
B5	<i>S. (B.) ourense</i> Watanabe and Nishino, 1995	Oura, Lake Biwa	52	IGPS No. 110213
S1	<i>Semisulcospira</i> (<i>Semisulcospira</i>) <i>libertina</i> (Gould, 1859)	Sendai, Miyagi	56	IGPS No. 110214
S2	<i>S. (S.) libertina</i> (Gould, 1859)	Fukushima, Fukushima	52	IGPS No. 110215
S3	<i>S. (S.) libertina</i> (Gould, 1859)	Sekikawa, Niigata	46	IGPS No. 110216
S4	<i>S. (S.) libertina</i> (Gould, 1859)	Fujieda, Shizuoka	52	IGPS No. 110217
S5	<i>S. (S.) libertina</i> (Gould, 1859)	Kanazawa, Ishikawa	52	IGPS No. 110218
S6	<i>S. (S.) libertina</i> (Gould, 1859)	On-na, Okinawa	32	IGPS No. 110219
S7	<i>S. (S.) reiniana</i> (Brot, 1876)	Hamamatsu, Shizuoka	39	IGPS No. 110220
S8	<i>S. (S.) reiniana</i> (Brot, 1876)	Komaki, Aichi	40	IGPS No. 110221
S9	<i>S. (S.) reiniana</i> (Brot, 1876)	Takatomi, Gifu	52	IGPS No. 110222
S10	<i>S. (S.) kurodai</i> Kajiyama and Habe, 1961	Yoka, Hyogo	60	IGPS No. 110223
M1	<i>Melanoides tuberculata</i> (Müller, 1774)	Nago, Okinawa	32	IGPS No. 110224

MATERIALS AND METHODS

Samples

Samples (*Semisulcospira* spp.) were collected between July 1999 and November 2002 from 21 sites on the main island of Japan (Honshu), including Lake Biwa and Okinawa Island (Fig. 1, Table 1). Thirty or more individuals were collected manually at each site within an area of approximately 10 m² on the bottom of a river or lake at a depth shallower than 1 m. The species *Melanoides tuberculata* (Müller, 1774) was collected in Nago, Okinawa Island (M1 in Fig. 1 and Table 1), and considered to be an outgroup of the genus *Semisulcospira* in the phylogenetic analysis.

Starch gel electrophoresis and gel staining

Starch gel electrophoresis and staining for allozyme analysis were performed as described by Fidhiany *et al.* (1988) and Shaw and Prasad (1970). Specimens were stored at -80 °C until dissection, and extracts from the digestive gland and foot were subjected to starch gel electrophoresis. Before gel electrophoresis, some pieces of digestive gland and foot were dissected, steeped in 0.5 M saccharose solution, and stored

overnight at -20 °C. For gel electrophoresis, the samples were defrosted and centrifuged at 12,000 rpm for 10 min at 1°C. The supernatant was absorbed on a small strip of filter paper (4 mm × 10 mm) and then inserted in the starch gel. Gel electrophoresis was performed under constant voltage (300 V) for five or more hours with a Tris-citrate (pH 7.0) buffer

Table 2. Enzymes examined and symbols for the gene loci encoding them. E.C., Enzyme Commission numbers.

Enzyme	E. C.	Locus
Malate Dehydrogenase (MDH)	1.1.1.37	<i>Mdh+</i> , <i>Mdh-</i>
Glucosephosphate Isomerase (GPI)	5.3.1.9	<i>Gpi</i>
Isocitrate Dehydrogenase (IDH)	1.1.1.42	<i>Idh</i>
Mannose-6-Phosphate Isomerase (MPI)	5.3.1.8	<i>Mpi</i>
Phosphoglucumutase (PGM)	2.7.5.1	<i>Pgm-1</i> , <i>Pgm-2</i>
6-Phosphoglucuronate Dehydrogenase (6PGD)	1.1.1.44	<i>6pgd</i>
Aspartate Aminotransferase (AAT)	2.6.1.1	<i>Aat</i>
Superoxide Dismutase (SOD)	1.15.1.1	<i>Sod</i>

system (Shaw and Prasad 1970). The procedure for gel staining followed Shaw and Prasad (1970). Ten enzyme loci were resolved from eight enzymes in this study (Table 2).

Data analysis

Genotype and allelic frequencies for the 10 loci were determined for all local populations. Genotype frequencies at polymorphic loci were examined for agreement with the expectations of Hardy-Weinberg equilibrium (HWE), using a Chi-square test. The genetic relationships among 24 populations were compared using Nei's genetic distance (D ; Nei 1972). The distance was used to cluster populations using the unweighted pair-group method with arithmetic means (UPGMA) algorithm (Sokal and Michener 1958), neighbor-joining method (Saitou and Nei 1987), and maximum likelihood (Felsenstein 1981). To investigate the robustness of nodes, we applied the bootstrap method with 1000 replicates (Felsenstein 1985). Calculation of genetic distances and construction of phylogenetic trees were performed using the PhylogenyInference Package (PHYLIP) version 3.6 (Felsenstein 2005) and trees were drawn using TREEVIEW (Page 1996).

Embryo and adult shell treatment

After dissection, embryos and adult shells were deposited in the Museum of Natural History, Tohoku University, with a reference number given for morphological identification and for voucher specimens (Table 1). The embryos and adult shells were soaked in sodium hypochlorite solution for several minutes to remove the remaining soft body tissue, washed in distilled water, and dried at room temperature. These shells were identified morphologically following the description of Davis (1969) and Watanabe and Nishino (1995).

RESULTS

Characteristics of loci

Ten gene loci for eight enzymes were detected. One or two bands were observed on the anodal side of the starch gel for the enzymes MPI and SOD. These band patterns are considered to be monomeric and controlled by a single locus, *Mpi* and *Sod*, respectively. A schema of the electrophoretic band patterns for MPI is shown (Fig. 2A). In the case of GPI, IDH, 6PGD, and AAT, one or three bands were observed on the anodal side, and the schematic band patterns of IDH are presented (Fig. 2B). These enzymes are considered to be dimeric. The enzyme MDH displayed two different band zones on the anodal and cathodal sides of the gel, and is considered to be controlled by two loci, *Mdh+* and *Mdh-*. The schematic band patterns of MDH are depicted (Fig. 2C). In *Mdh+*, one or two bands were observed on the anodal side,

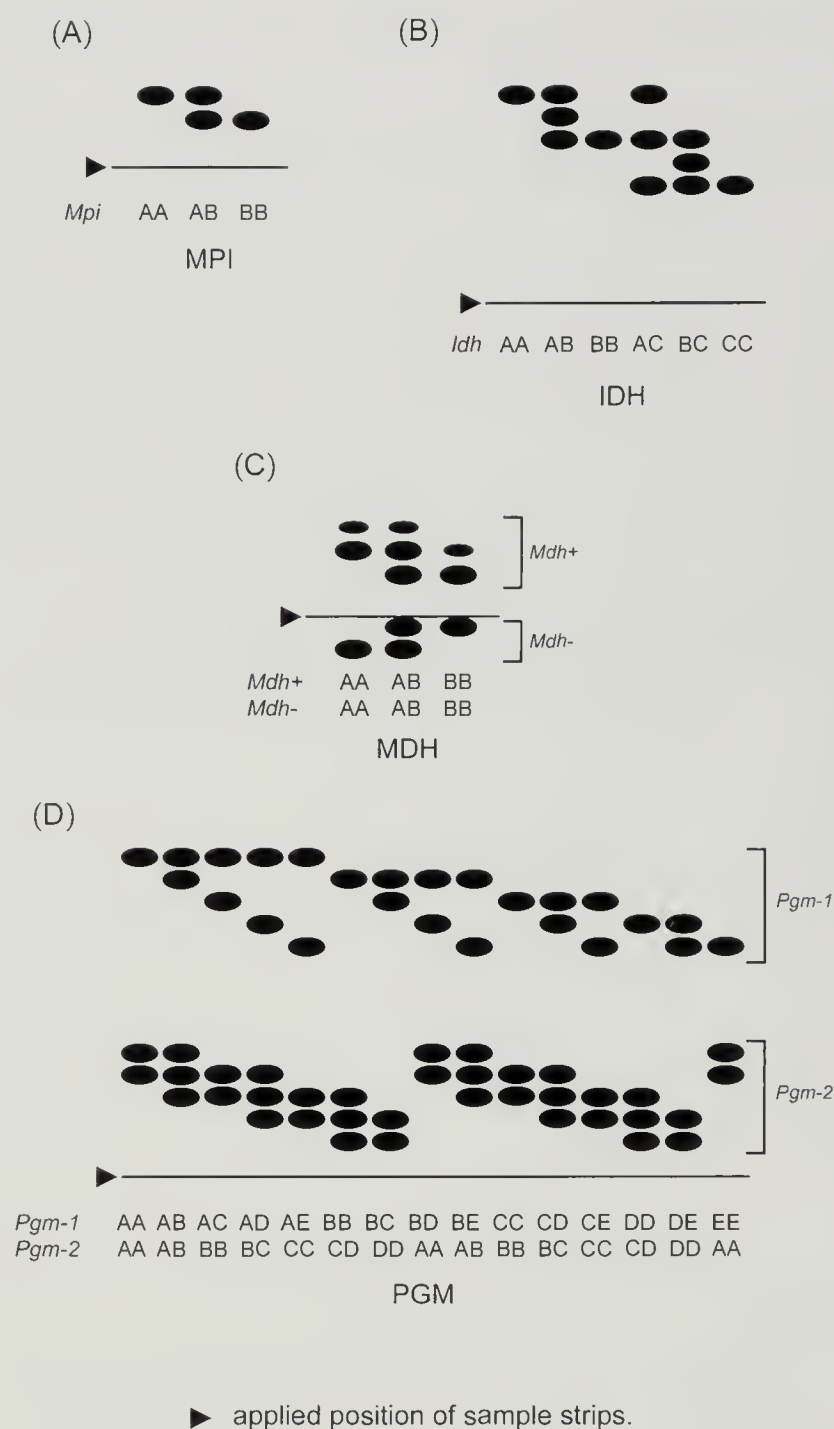


Figure 2. Schematic electrophoretic patterns of polymorphic enzymes. A, Mannose-6-phosphate isomerase (MPI); B, Isocitrate Dehydrogenase (IDH); C, Malate Dehydrogenase (MDH); D, Phosphoglucomutase (PGM).

accompanied occasionally by a pale additional “ghost” band. Therefore, *Mdh+* was presumed to be monomeric. *Mdh-* exhibited a one- or two-banded phenotype on the cathodal side of the gel and is also monomeric. In the case of PGM, two separate migrating zones were observed on the anodal side of the gel. The schematic band patterns of PGM are shown (Fig. 2D). These two migrating zones are controlled by two loci, *Pgm-1* and *Pgm-2*, respectively. The locus *Pgm-1* displayed one- or two-banded patterns and is considered to be monomeric. However, the locus *Pgm-2* showed two- or, rarely,

three-banded patterns. Because the two-banded pattern may be interpreted as a homozygote and the three-banded pattern as a heterozygote, the locus *Pgm-2* is presumed to be monomeric.

Genetic features of each species

Based on the phenotypes of each locus, the genotype of each individual was interpreted and the allelic frequencies of each population were calculated (Table 3).

The allelic frequencies of six Lake Biwa endemics, *Semisulcospira* (*Biwamelania*) *arenicola*, *S. (B.) decipiens*, *S. (B.) fluvialis*, *S. (B.) multigranosa*, *S. (B.) nakasekoe*, and *S. (B.) ourense*, were similar to each other. At eight loci (*Mdh+*, *Mdh-*, *Gpi*, *Idh*, *Mpi*, *6Pgd*, *Pgm-2*, and *Sod*), the most frequent allele was common among all populations of these species. Especially at loci *Gpi*, *6Pgd*, and *Pgm-2*, alleles C, D, and C were predominant, respectively, and these alleles were virtually fixed in those six species. The allelic frequencies at the remaining two loci (*Pgm-1* and *Aat*) were somewhat diversified. Allele B was frequent at *Pgm-1*, and allele C was also common in all populations of these species. At the locus *Aat*, allele B was predominant, and allele A was not uncommon.

Semisulcospira (*Biwamelania*) *niponica* (ribbed type), *S. (B.) niponica* (nodose type), and *S. (B.) fuscata* are also indigenous to Lake Biwa. Alleles *Mdh-*, *Idh*, *Mpi*, and *Sod* in these species were almost fixed, as in the other six Lake Biwa endemics mentioned above, but the most fixed alleles at loci *Gpi*, *6Pgd*, and *Pgm-2*—alleles B, B and A—are quite different from those of the six Lake Biwa endemics. At loci *Aat* and *Pgm-1*, the alleles were almost fixed to B and B, respectively. At the locus *Mdh+*, allele B was predominant in the populations of *S. (B.) niponica* (nodose type) and *S. (B.) fuscata*, but allele A was more abundant in the population of *S. (B.) niponica* (ribbed type).

Semisulcospira (*Biwamelania*) *habei*, another Lake Biwa endemic, had genetic features similar to *S. (S.) kurodai*, which is found around Kinki district (in the central area of Japan) outside Lake Biwa. The alleles at the seven loci *Mdh-*, *Gpi*, *Mpi*, *6Pgd*, *Pgm-2*, *Aat*, and *Sod* were commonly fixed in the populations of the two species, as in the Lake Biwa species *S. (B.) niponica* (ribbed type), *S. (B.) niponica* (nodose type), and *S. (B.) fuscata* mentioned above. However, allele A in the locus *Idh* was frequently found in the populations of *S. (B.) habei* and *S. (S.) kurodai*, and characteristically observed in these two species. Allele B was abundant at the locus *Mdh+* of these two species, but allele A also existed in the populations of *S. (B.) habei*. The analysis of locus *Pgm-1* was complicated. Although allele B at this locus was fixed in the population of *S. (S.) kurodai*, alleles B and D coexisted in the populations of *S. (B.) habei*. Allele B was predominant in population B1 of

S. (B.) habei, and allele D was abundant in population B2 of the species.

The allelic frequencies of the species *Semisulcospira* (*S.*) *libertina*, which inhabits river systems throughout the Japanese Islands, were highly variable among populations, except for the loci *Mdh+*, *Mdh-*, *Mpi*, *Aat*, and *Sod*. Allele B at the locus *Gpi* was abundant in the populations, except for population S4 of this species; allele C was also common in population S4. Allele B was frequent at locus *Idh*, but alleles C and A also occurred at low frequency in population S3 and S5, respectively. The allelic frequencies of loci *6Pgd*, *Pgm-1*, and *Pgm-2* were rather variable, and the most frequent allele differed by population of the species.

The genetic characterization of the species *Semisulcospira* (*S.*) *reiniana* was rather simple, except for loci *6Pgd* and *Pgm-1*. Alleles A, B, C, or D were recognized in *6Pgd*; C was the most abundant allele. The locus *Pgm-1* was almost fixed to allele B in populations S7 and S8 of this species, but alleles C and D were also common in populations S8 and S9. Allele B at locus *Mpi* was specific to populations of *S. (S.) reiniana*, whereas allele A was almost fixed in the other Japanese species examined in this study.

Genetic relationship among species and HWE test

Six Lake Biwa endemics, *Semisulcospira* (*Biwamelania*) *arenicola*, *S. (B.) decipiens*, *S. (B.) fluvialis*, *S. (B.) multigranosa*, *S. (B.) nakasekoe*, and *S. (B.) ourense*, formed a definite cluster with small *D* values ranging from 0.0009 to 0.0186 in all trees (Figs. 3-5, Table 4). These species were well discriminated from other species and their monophyly was indicated by high bootstrap values in all trees (Figs. 3-5).

Other Lake Biwa endemics, *Semisulcospira* (*Biwamelania*) *niponica* (ribbed type), *S. (B.) niponica* (nodose type), and *S. (B.) fuscata* were in the same clade with low bootstrap support in the UPGMA and maximum likelihood trees (Figs. 3, 5), but they were not in the distinct clade in the neighbor-joining tree (Fig. 4). *S. (B.) niponica* and *S. (B.) fuscata* were seen in the paraphyletic group to the *S. (B.) habei* group with low bootstrap support in the neighbor-joining tree (Fig. 4). *S. (B.) habei*, another Lake Biwa species, clustered with the non-Biwa species *S. (S.) kurodai* with *D* values ranging from 0.0053 to 0.0399 and with a high bootstrap support in all trees (Figs. 3-5).

Most of the eight polymorphic loci of the Lake Biwa populations did not deviate significantly from HWE, except for *Aat* of *Semisulcospira* (*Biwamelania*) *arenicola* (B8) and *Pgm-1* of *S. (B.) fluvialis* (B9) and *S. (B.) habei* (B1) (level of significance $\alpha = 0.05$). The observed genotype frequencies among the Lake Biwa populations were similar to each other within each above-mentioned cluster. There were no significant differences of the observed genotype frequencies even

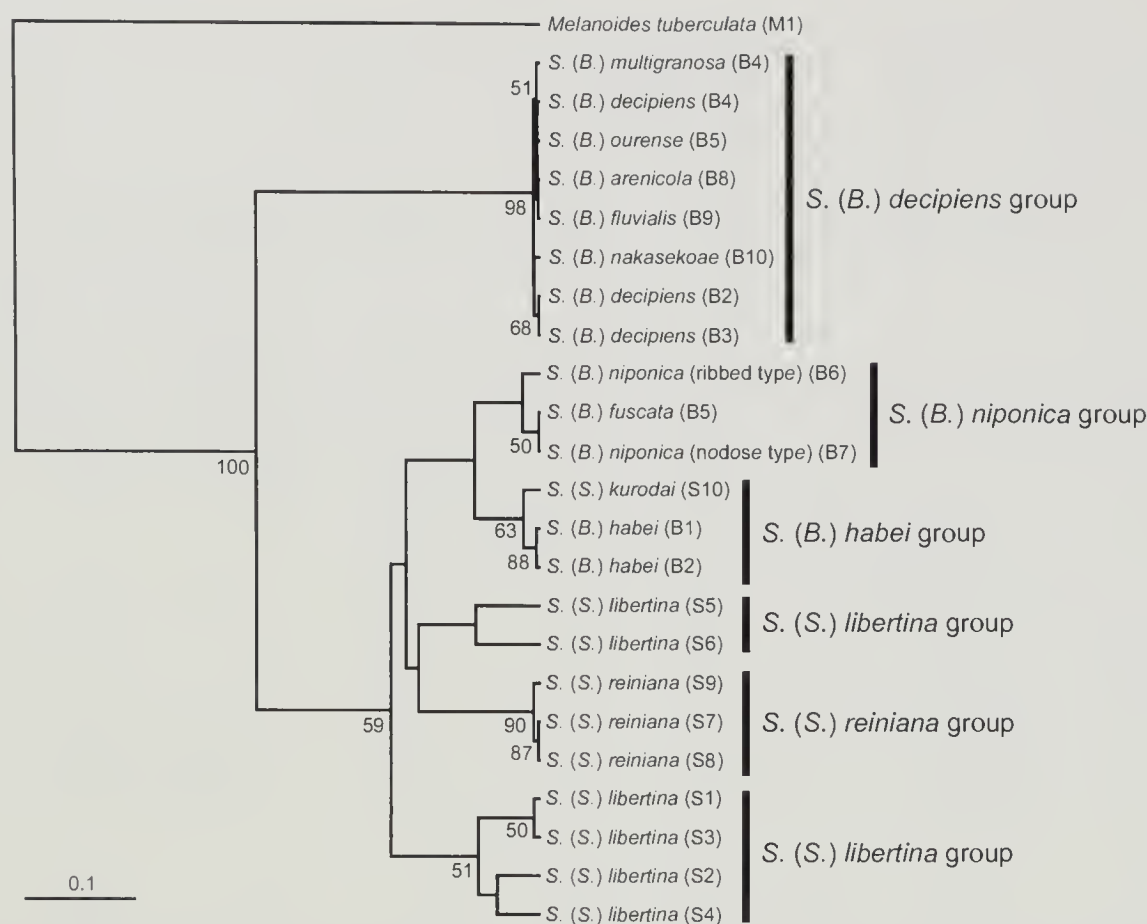


Figure 3. Relationships of *Semisulcospira* populations studied as shown in a UPGMA dendrogram. Numbers on the branches are the percentage of 1000 bootstrap replicates ($\geq 50\%$). See Fig. 1 for the sample collection sites in the parenthesis.

between *S. (B.) multigranosa* and *S. (B.) decipiens* populations inhabiting the same site, B4.

The populations of *Semisulcospira (S.) libertina* did not form a distinct cluster in all trees, with relatively large *D* values ranging from 0.0097 to 0.3963 (Figs. 3-5). Populations S5 and S6 were divergent from the cluster of populations S1, S2, S3, and S4 of *S. (S.) libertina*. The cluster of populations S5 and S6 was rather closely related to the cluster of *S. (S.) reiniana* without a significant support value in UPGMA and the maximum likelihood trees (Figs. 3, 5). Populations S7, S8, and S9 of *S. (S.) reiniana* clustered together with high bootstrap values in all trees, and their *D* values were small, ranging from 0.0010 to 0.0116 (Figs. 3-5).

DISCUSSION

Based on the genetic features and the topologies of all of the phylogenetic trees, the 23 populations examined in the present study were clustered robustly into two major groups, the *Semisulcospira (Biwamelania) decipiens* group and non-*decipiens* group (Figs. 3-5). The non-*decipiens* group could be separated into four subclusters, the *S. (B.) niponica*, *S. (B.)*

habei, *S. (S.) libertina*, and *S. (S.) reiniana* groups, although bootstrap support values among populations of these groups were not necessarily high.

The *Semisulcospira (Biwamelania) decipiens* group consists of populations of *S. (B.) arenicola*, *S. (B.) decipiens*, *S. (B.) fluvialis*, *S. (B.) multigranosa*, *S. (B.) nakasekoe*, and *S. (B.) ourense*, which are endemic species of Lake Biwa, with extremely high bootstrap support in all phylogenetic trees (Figs. 3-5). The most abundant alleles in this group are nearly fixed at loci *Gpi*, *6Pgd*, and *Pgm-2*; these loci can be used to discriminate the group from other groups (Table 3). The *D* values of each pair between species of the *S. (B.) decipiens* group and those of other groups are significantly large, averaging 0.4439 or greater (Table 5). In contrast, the *D* values of each pair of populations within the *S. (B.) decipiens* group are conspicuously small, averaging 0.0074 (Table 5). Thus, the genetic features of the *S. (B.) decipiens* group are distinct from those of any other group.

The *Semisulcospira (Biwamelania) habei* group is composed of populations of *S. (B.) habei* and *S. (S.) kurodai* with moderately high bootstrap support values; the former is a Lake Biwa species, and the latter a non-Biwa species. The *D*

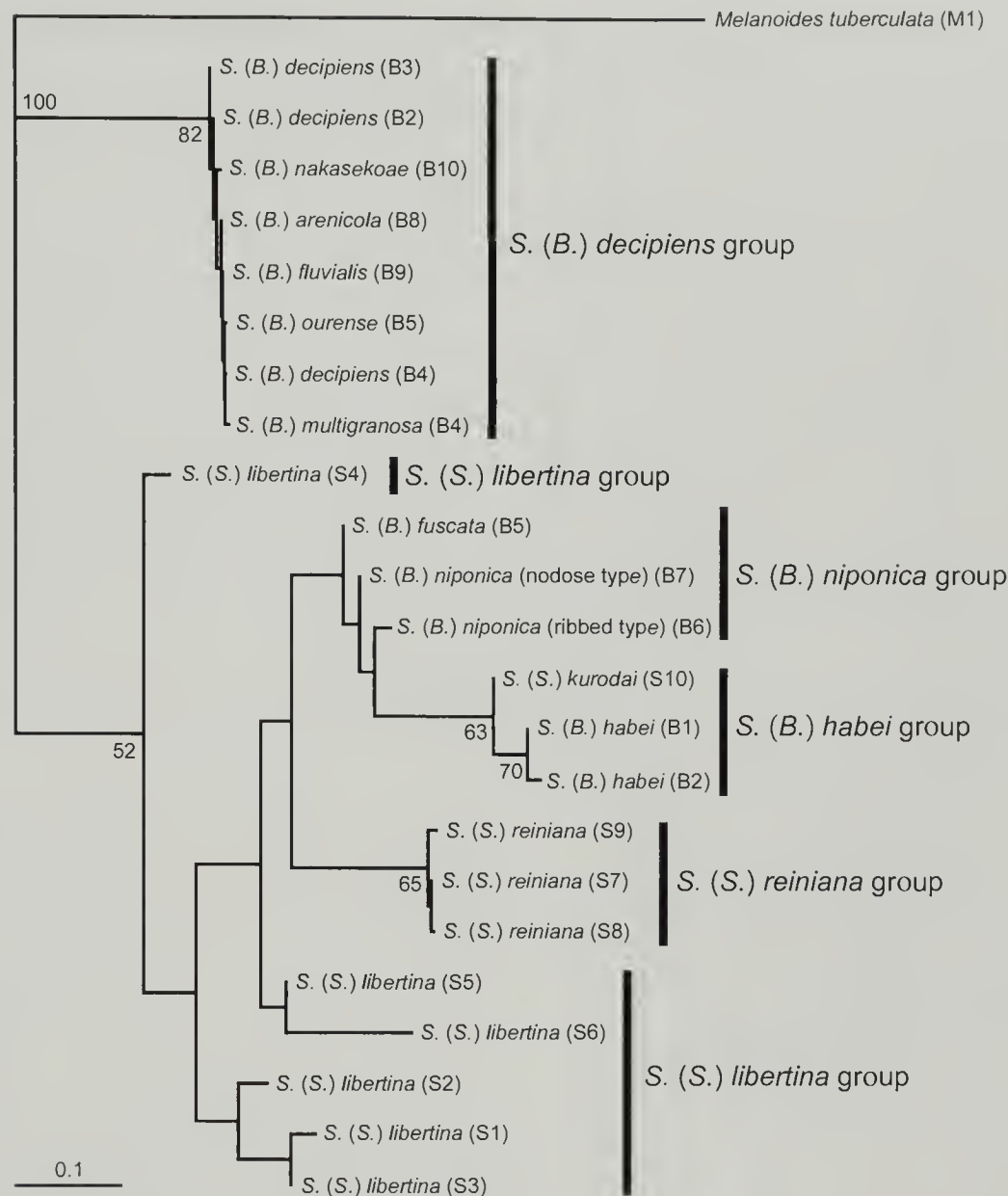


Figure 4. Relationships of *Semisulcospira* populations studied as shown in a neighbor-joining dendrogram. Numbers on the branches are the percentage of 1000 bootstrap replicates ($\geq 50\%$). See Fig. 1 for the sample collection sites in the parenthesis.

values among populations of the *S. (B.) habei* group are also small, averaging 0.0211 (Table 5). The populations of this group form a single clade in all phylogenetic trees with moderately high bootstrap support (Figs. 3-5). Species of this group are closely related to those of the *S. (B.) niponica* group, with an average *D* value of 0.1192 (Table 5).

The *Semisulcospira* (*Biwamelania*) *niponica* group is composed of populations of *S. (B.) niponica* (ribbed type), *S. (B.) niponica* (nodose type), and *S. (B.) fuscata*, which are also Lake Biwa endemics, without significant support values. The *D* values among populations of the *S. (B.) niponica* group are small, with an average of 0.0204 (Table 5); these populations form a monophyletic group in UPGMA and maximum likelihood trees (Figs. 3, 5). In the neighbor-joining tree, these populations are not positioned within a distinct clade, but are closely related to the *S. (B.) habei* group (Fig. 4).

The genetic characteristics of the *Semisulcospira* (*Biwamelania*) *niponica* and *S. (B.) habei* groups are radically different from those of the *S. (B.) decipiens* group. The most abundant alleles at loci *Gpi*, *6Pgd*, and *Pgm-2* of the former two groups are entirely distinct from those of the latter *S. (B.) decipiens* group, and the mean genetic distances between the *S. (B.) niponica* or *S. (B.) habei* groups and the *S. (B.) decipiens* group are extremely large, 0.4780 or 0.6538, respectively (Table 5). In contrast, the mean genetic distances between *S. (B.) niponica* or *S. (B.) habei* groups and non-Biwa *S. (S.) libertina* or *S. (S.) reiniana* groups are rather small, 0.3097 or less (Table 5).

The populations of the *Semisulcospira* (*S.*) *reiniana* group, which are non-Biwa species, form a single cluster with moderately high bootstrap support in all phylogenetic trees (Figs. 3-5). The *S. (S.) reiniana* group is well discriminated from any other group on the basis of the allele at the locus

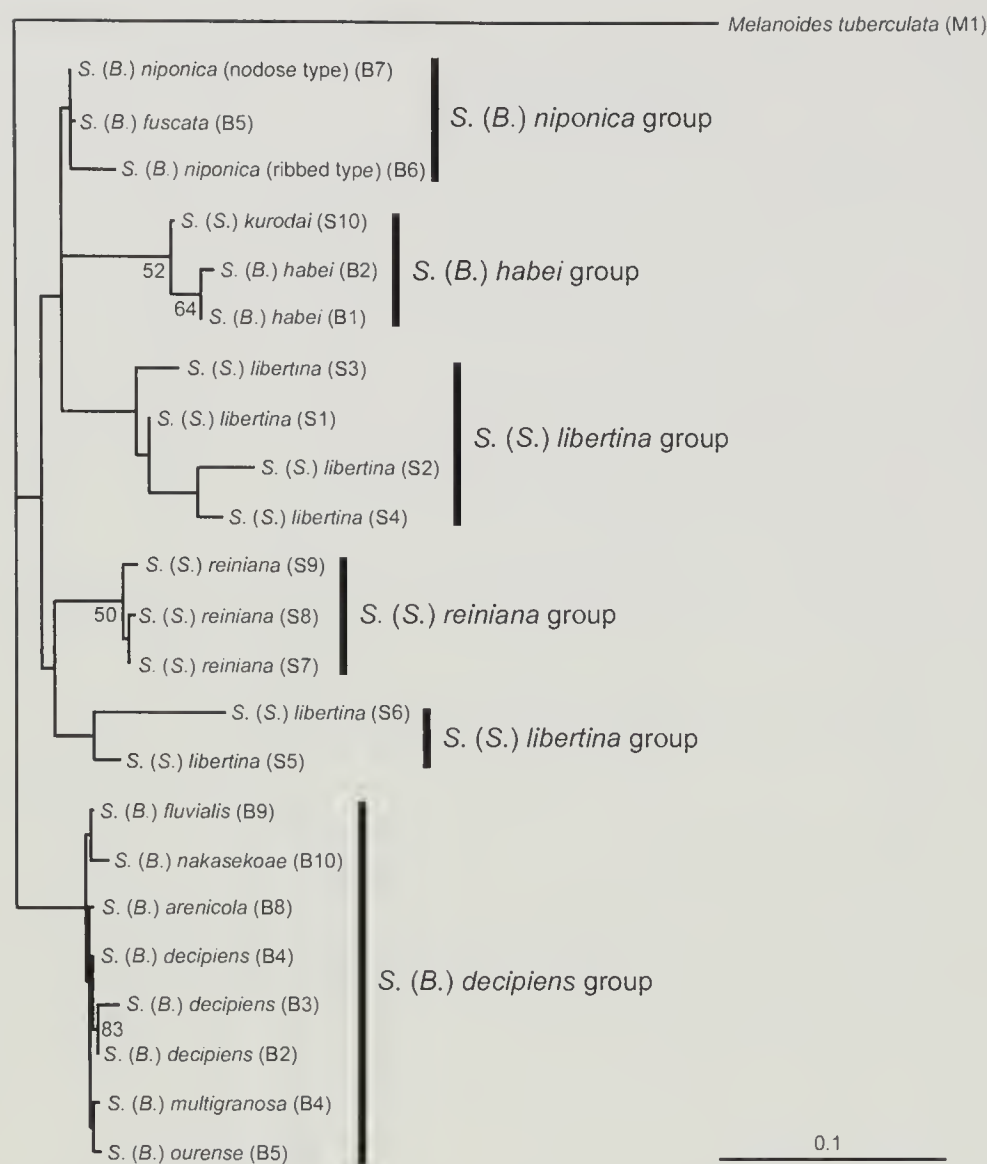


Figure 5. Relationships of *Semisulcospira* populations studied as shown in a maximum likelihood dendrogram. Numbers on the branches are the percentage of 1000 bootstrap replicates ($\geq 50\%$). See Fig. 1 for the sample collection sites in the parenthesis.

MPI. Allele B at *Mpi* is fixed only in the populations of the *S. (S.) reiniana* group, whereas allele A is fixed in the populations of the other groups. The mean genetic distance among populations of the *S. (S.) reiniana* group, 0.0075, is fairly small (Table 5), although this group is distributed widely in the Kanto district and to the west in Japan.

Populations of the non-Biwa *Semisulcospira* (*S. (S.) libertina* group did not form a distinct cluster with low bootstrap supports in all phylogenetic trees (Figs. 3- 5). Populations of the *S. (S.) libertina* group are distributed widely throughout Japan and exhibit the greatest genetic variability among all groups of the genus *Semisulcospira*. The mean genetic distance among populations of the *S. (S.) libertina* group, 0.1750, is remarkably large (Table 5).

As mentioned above, populations of the *Semisulcospira* (*Biwamelania*) *decipiens* group are well discriminated from any other populations examined in the present study. The *S.*

(*B.*) *decipiens* group formed a definite cluster with high bootstrap support in all trees (Figs. 3-5), and, therefore, exhibit genetic features that are distinct from those of any other group. In contrast, the *D* values of each pair of populations within the *S. (B.) decipiens* group, ranging from 0.0009 to 0.0186, are conspicuously small (Table 4), and the allelic frequencies of eight populations of the *S. (B.) decipiens* group are similar to each other.

Most of the eight polymorphic loci of the 14 populations did not deviate significantly from HWE, except for *Aat* of *Semisulcospira* (*Biwamelania*) *arenicola* (B8) and *Pgm-1* of *S. (B.) fluvialis* (B9) and *S. (B.) habei* (B1) (level of significance $\alpha = 0.05$). The observed genotype frequencies of the populations of the *S. (B.) decipiens* group were very similar to each other. There were no significant differences of the observed genotype frequencies even between *S. (B.) multigranosa* and *S. (B.) decipiens* populations inhabiting the

same site B4. Based on the similar genotype frequencies and the small genetic distances, it is difficult to determine whether the eight populations of the *S. (B.) decipiens* group examined in this study are samples from a single conspecific population. However, Burch (1968) and Kobayashi (1986) have reported differences in the chromosome numbers among some Japanese *Semisulcospira* species. Burch (1968) reported that *S. (B.) multigranosa*, *S. (B.) decipiens*, *S. (B.) habei*, *S. (B.) niponica*, and *S. (S.) kurodai* have chromosome numbers of $2n = 28$ to 31 ; $2n = 25$ or 26 ; $2n = 17$ to 20 ; $2n = 25$ to 27 ; and $2n = 35$ or 36 , respectively. Kobayashi (1986) reported that *S. (B.) nakasekoe* has a chromosome number of $2n = 38$. Moreover, remarkable interspecific differences in shell morphology have been recognized among the species of the *S. (B.) decipiens* group (Davis 1969, Watanabe and Nishino 1995). Therefore, the eight populations of the *S. (B.) decipiens* group examined in this study may not be samples from a single conspecific population; their conspicuously small *D* values may suggest that speciation has begun.

Davis (1969) studied the taxonomy of Japanese *Semisulcospira* species and divided them into two groups: the *S. libertina* species complex and *S. niponica* species complex, based on the number of embryonic shells, basal cords of adult shells, and chromosomes. The *S. libertina* species complex contained *S. libertina*, *S. reiniana*, and *S. kurodai*, which occupy a wide area around Lake Biwa and throughout Japan. The *S. niponica* species complex was composed of six Lake Biwa endemic species: *S. niponica*, *S. decipiens*, *S. multigranosa*, *S. reticulata*, *S. nakasekoe*, and *S. habei*. Habe (1978) classified the “*S. niponica* species complex” under the genus *Biwamelania*, and Matsuoka and Nakamura (1981) assigned it to the subgenus *Biwamelania* with *S. niponica* as a holotype. The species of the *S. libertina* species complex have a chromosome number of $2n = 36$ (Burch 1968, Nakano *et al.* 1994), and species of the *S. niponica* species complex have fewer chromosomes, $2n = 17$ to 31 (Burch 1968). The former species are characterized by having seven or more basal cords of adult shells and 100 or more embryonic shells in the female brood pouch; the latter species are characterized by having from two to six basal cords and fewer embryonic shells (Davis 1969). Although the *S. (S.) kurodai* was placed in the *S. libertina* species complex by Davis (1969), the species was considered transitional between the two species complexes, as the adult shells have an average 5.1 basal cords and about 50 embryos per female brood pouch. All phylogenetic trees in the present study (Figs. 3-5) support the arguments of Davis (1969). Our allozyme data suggest that the *S. (S.) kurodai* relates intimately to not only the *S. (B.) habei* and *S. (B.) niponica* groups, especially to *S. (B.) habei*, but also *S. (S.) libertina* and *S. (S.) reiniana*.

Matsuoka (1987) presumed *Semisulcospira (Biwamelania) habei* to be the oldest extant species in Lake Biwa. Moreover, Nishino and Watanabe (2000) considered *S. (B.) habei* to be a common ancestor of all extant Lake Biwa species, with all extant Lake Biwa species derived from *S. (B.) habei* over a short period of time. As mentioned, *S. (B.) habei* was not positioned in a basal clade in all phylogenetic trees in the present study (Figs. 3-5), and *S. (B.) habei* was found to be related intimately to both the Lake Biwa *S. (B.) niponica* group and the non-Biwa *S. (S.) libertina* and *S. (S.) reiniana* groups. In contrast, the Lake Biwa *S. (B.) decipiens* group was found to be genetically distant from the other Lake Biwa taxa including *S. (B.) habei*. Therefore, the notion that *S. (B.) habei* is the oldest common ancestor of all extant Lake Biwa species should be reconsidered.

Recently, Lee *et al.* (2007) investigated the population genetic structure and phylogenetic relationships of four morphospecies of *Semiculcospira* sampled from multiple South Korean drainages. They reported a taxonomically heterogeneous species complex containing population-level admixtures of genotypes, and they considered that it might be attributed to a number of generative mechanisms, such as ancestral polymorphisms, hybridization, and the existence of cryptic species. In the present study, several *Semiculcospira* species did not form a robust monophyletic clade, except *S. (B.) habei* and *S. (S.) reiniana* (Figs. 3-5). As pointed out by Lee *et al.* (2007), comprehensive phylogenetic studies and research on ecophenotypic plasticity in shell morphology of the Japanese *Semiculcospira* species may be needed in future.

CONCLUSION

We investigated the genetic variations of 12 living Japanese species of the gastropod genus *Semisulcospira*, including 9 Lake Biwa endemics, based on allozyme analysis. The Lake Biwa endemics were genetically discriminated into three groups, the *S. (Biwamelania) decipiens*, *S. (B.) niponica*, and *S. (B.) habei* groups. The genetic features of six Lake Biwa species of the *S. (B.) decipiens* group are distinct from those of any other *Semisulcospira* species examined in the present study. In contrast, other Lake Biwa species of the *S. (B.) niponica* and *S. (B.) habei* groups are closely related to each other and to the non-Biwa species, *S. (S.) libertina* and *S. (S.) reiniana*. Our allozyme data suggest that the Japanese *Semisulcospira* species are distinguished robustly into two major lineages, a *S. (B.) decipiens* group and a separate non-decipiens group, and that all extant Lake Biwa endemics do not form a single species flock, as proposed previously.

Table 4. Nei's genetic distances (*D*) among *Semisulcospira* populations. See Fig. 1 for the sample collection sites in the parenthesis. Numbers (1 to 24) indicate local population.

Population	1	2	3	4	5	6	7	8	9	10	11
1: <i>S. (B.) arenicola</i> (B8)											
2: <i>S. (B.) decipiens</i> (B2)	0.0059										
3: <i>S. (B.) decipiens</i> (B3)	0.0062	0.0009									
4: <i>S. (B.) decipiens</i> (B4)	0.0028	0.0045	0.0072								
5: <i>S. (B.) fluvialis</i> (B9)	0.0018	0.0088	0.0099	0.0041							
6: <i>S. (B.) multigranosa</i> (B4)	0.0055	0.0114	0.0150	0.0036	0.0051						
7: <i>S. (B.) nakasekoe</i> (B10)	0.0061	0.0098	0.0081	0.0116	0.0052	0.0186					
8: <i>S. (B.) ourense</i> (B5)	0.0029	0.0130	0.0153	0.0034	0.0036	0.0046	0.0126				
9: <i>S. (B.) fuscata</i> (B5)	0.4462	0.4712	0.4578	0.4543	0.4395	0.4777	0.4237	0.4424			
10: <i>S. (B.) niponica</i> (ribbed type) (B6)	0.5163	0.5477	0.5302	0.5275	0.5106	0.5523	0.4936	0.5101	0.0337		
11: <i>S. (B.) niponica</i> (nodose type) (B7)	0.4526	0.4804	0.4663	0.4618	0.4461	0.4844	0.4307	0.4479	0.0008	0.0269	
12: <i>S. (B.) habei</i> (B1)	0.6611	0.6824	0.6644	0.6719	0.6558	0.6986	0.6314	0.6501	0.1152	0.1386	0.1079
13: <i>S. (B.) habei</i> (B2)	0.6893	0.7018	0.6832	0.6993	0.6836	0.7284	0.6560	0.6829	0.1236	0.1450	0.1182
14: <i>S. (S.) kurodai</i> (S10)	0.5989	0.6348	0.6180	0.6134	0.5937	0.6321	0.5758	0.5849	0.0959	0.1400	0.0883
15: <i>S. (S.) libertina</i> (S1)	0.4452	0.4635	0.4498	0.4521	0.4395	0.4778	0.4207	0.4455	0.1226	0.1698	0.1250
16: <i>S. (S.) libertina</i> (S2)	0.4472	0.4309	0.4220	0.4336	0.4424	0.4832	0.4127	0.4475	0.2266	0.2860	0.2352
17: <i>S. (S.) libertina</i> (S3)	0.4124	0.4373	0.4244	0.4193	0.4063	0.4441	0.3904	0.4076	0.0827	0.1300	0.0839
18: <i>S. (S.) libertina</i> (S4)	0.3339	0.3470	0.3463	0.3216	0.3265	0.3615	0.3128	0.3183	0.2946	0.3442	0.2979
19: <i>S. (S.) libertina</i> (S5)	0.4427	0.4574	0.4499	0.4321	0.4363	0.4742	0.4200	0.4274	0.1100	0.1481	0.1111
20: <i>S. (S.) libertina</i> (S6)	0.5971	0.5653	0.5641	0.5648	0.5884	0.6332	0.5554	0.5777	0.2454	0.2985	0.2579
21: <i>S. (S.) reiniana</i> (S7)	0.5786	0.6104	0.5962	0.5854	0.5735	0.6178	0.5511	0.5712	0.1629	0.2110	0.1652
22: <i>S. (S.) reiniana</i> (S8)	0.5855	0.6143	0.5999	0.5917	0.5805	0.6255	0.5567	0.5794	0.1671	0.2167	0.1702
23: <i>S. (S.) reiniana</i> (S9)	0.5858	0.6020	0.5879	0.5895	0.5803	0.6263	0.5528	0.5847	0.1828	0.2372	0.1888
24: <i>M. tuberculata</i> (M1)	0.8327	0.8030	0.8011	0.8068	0.8528	0.8817	0.8332	0.8288	1.0904	1.0527	1.0996

Table 5. Mean genetic distances between *Semisulcospira* species groups. Numbers (1 to 5) indicate species group.

Species group	1	2	3	4	5
1: <i>S. (B.) decipiens</i> group	0.0074				
2: <i>S. (B.) habei</i> group	0.6538	0.0211			
3: <i>S. (B.) niponica</i> group	0.4780	0.1192	0.0204		
4: <i>S. (S.) libertina</i> group	0.4439	0.3097	0.1983	0.1750	
5: <i>S. (S.) reiniana</i> group	0.5886	0.3115	0.1891	0.2865	0.0075

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Table 4. (Continued)

12	13	14	15	16	17	18	19	20	21	22	23	24
0.0053												
0.0180	0.0399											
0.2644	0.2735	0.2357										
0.3655	0.3749	0.3581	0.0794									
0.2058	0.2212	0.1735	0.0097	0.0823								
0.4639	0.5056	0.4203	0.1525	0.0776	0.1340							
0.2102	0.2406	0.1880	0.2495	0.1784	0.1756	0.1533						
0.3491	0.3532	0.3716	0.3963	0.1973	0.3320	0.2897	0.1170					
0.3143	0.3397	0.2760	0.3142	0.3357	0.2527	0.3441	0.1537	0.2994				
0.3156	0.3377	0.2817	0.3199	0.3320	0.2585	0.3499	0.1563	0.2945	0.0010			
0.3127	0.3206	0.3056	0.3390	0.3291	0.2804	0.3647	0.1644	0.2679	0.0116	0.0099		
1.3673	1.3682	1.4380	1.0910	0.8042	1.0613	0.7826	0.8313	0.8109	0.9285	0.9270	0.8921	

LITERATURE CITED

- Burch, J. B. 1968. Cytotaxonomy of some Japanese *Semisulcospira* (Streptoneura: Pleuroceridae). *Journal of Conchylology* 107: 3-51.
- Davis, G. M. 1969. A taxonomic study of some species of *Semisulcospira* in Japan. *Malacologia* 7: 211-294.
- Felsenstein, J. 1981. Evolutionary tree from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution* 17: 368-376.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using bootstrap. *Evolution* 39: 783-791.
- Felsenstein, J. 2005. *PHYLIP (Phylogeny Inference Package) version 3.6*. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- Fidhiany, L., A. Kijima, and Y. Fujio. 1988. Genetic divergence between two types in *Palaemon paucidens*. *Tohoku Journal of Agricultural Research* 39: 39-45.
- Habe, T. 1978. Freshwater mollusks in Korea and Japan as the intermediate hosts of Trematodes. *Yonsei Reports on Tropical Medicine* 9: 91-96.
- Kamiya, S. and M. Shimamoto. 2005. Genetic and morphological variations of two freshwater snails, *Semisulcospira libertina* and *S. reiniana* in Japan. *Venus* 64: 161-176 [In Japanese with English abstract].
- Kobayashi, T. 1986. Karyotypes of four species of the Genus *Semisulcospira* in Japan. *Venus* 45: 127-137 [In Japanese with English abstract].
- Lee, T., H. C. Hong, J. J. Kim, and D. O. Foighil. 2007. Phylogenetic and taxonomic incongruence involving nuclear and mitochondrial markers in Korean populations of the freshwater snail genus *Semisulcospira* (Cerithioidea: Pleuroceridae). *Molecular Phylogenetics and Evolution* 43: 386-397.
- Matsuoka, K. 1987. Malacofaunal succession in Pliocene to Pleistocene non-marine sediments in the Omi and Ueno basins, central Japan. *The Journal of Earth Sciences, Nagoya University* 35: 23-115.
- Matsuoka, K. and T. Nakamura. 1981. Preliminary report of the freshwater mollusks from the Pleistocene Katata formation of the Kobiwako group in Shiga prefecture, Japan. *Bulletin of the Mizunami Fossil Museum* 8: 105-126 [In Japanese with English abstract].

- Mori, S. and T. Miura. 1980. List of plant and animal species living in Lake Biwa. *Memoirs of the Faculty of Science, Kyoto University. Series of Biology* **8**: 1-33.
- Nakano, D., K. Wada, and K. Izawa. 1994. Karyotype of the freshwater snail, *Semisulcospira reiniana* (Prosobranchia: Pleuroceridae). *Venus* **53**: 43-47 [In Japanese with English abstract].
- Nei, M. 1972. Genetic distance between populations. *American Naturalist* **106**: 283-292.
- Nishino, M. and N. Watanabe. 2000. Evolution and endemism in Lake Biwa with special reference to its gastropod mollusc fauna. *Advances in Ecological Research* **31**: 151-180.
- Oniwa, K. and M. Kimura. 1986. Genetic variability and relationships in six snail species of the genus *Semisulcospira*. *The Japanese Journal of Genetics* **61**: 503-514.
- Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357-358.
- Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.
- Shaw, C. R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. *Biochemical Genetics* **4**: 297-320.
- Sokal, R. R. and C. D. Michener. 1958. A statistical method for evaluating systematic relationships. *The University of Kansas Scientific Bulletin* **28**: 1409-1439.
- Urabe, M. 1992. A discrimination and morphological comparison of two snail species of the genus *Semisulcospira* in a single river. *Venus* **50**: 276-286 [In Japanese with English abstract].
- Watanabe, N. 1984. Studies on taxonomy and distribution of the freshwater snails, genus *Semisulcospira* in the three islands inside Lake Biwa. *Japanese Journal of Limnology* **45**: 194-203.
- Watanabe, N. and M. Nishino. 1995. A study on taxonomy and distribution of the freshwater snail, genus *Semisulcospira* in Lake Biwa, with descriptions of eight new species. *Lake Biwa Study Monographs* **6**: 1-33.

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